

ENHANCED ACCUMULATION OF TREHALOSE IN PLANTS

FIELD OF THE INVENTION

The invention relates to a method for the production of trehalose
5 in plant cells, and plants. The invention is particularly related to a
method for increasing the levels of trehalose accumulation in plants
capable of producing trehalose. The invention further comprises higher
plants, preferably *Angiospermae*, and parts thereof, which as a result of
such methods, contain relatively high levels of trehalose. The invention
10 further relates to plant cells, plants or parts thereof according to the
invention obtained after processing thereof.

STATE OF THE ART

Trehalose is a general name given to D-glucosyl D-glucosides which
15 comprise disaccharides based on two α -, α , β - and β , β -linked glucose
molecules. Trehalose, and especially α -trehalose
1-(O- α -D-glucopyranosyl)-1'-O- α -D-glucopyranose) is a widespread
naturally occurring disaccharide. However, trehalose is not generally
found in plants, apart from a few exceptions, such as the plant species
20 *Selaginella lepidophylla* (*Lycophyta*) and *Myrothamnus flabellifolia*. Apart
from these species, trehalose is found in root nodules of the *Leguminosae*
(*Spermatophytae*, *Angiospermae*), wherein it is synthesized by bacteroids;
the trehalose so produced is capable of diffusing into the root cells.
Apart from these accidental occurrences, plant species belonging to the
25 *Spermatophyta* apparently lack the ability to produce and/or accumulate
trehalose.

In International patent application WO 95/01446, filed on June 30,
1994 in the name of MOGEN International NV, a method is described for
providing plants not naturally capable of producing trehalose with the
30 capacity to do so. The method comprises introducing into the cells of
said plants a recombinant polynucleotide encoding trehalose phosphate
synthase under the control of regulatory elements necessary for
expression of said recombinant DNA in plant cells. In one embodiment,
tobacco and potato plants had been transformed with a recombinant
35 polynucleotide encoding TPS from *E. coli*, under the control of the CaMV
35S RNA promoter. Levels of trehalose accumulation in these plants tended

to be rather low.

In spite of the absence of trehalose as a substrate in most higher plant species, the occurrence of trehalose-degrading activity has been reported for a considerable number of higher plant species, including those known to lack trehalose. The responsible activity could be attributed to a trehalase enzyme.

Reports suggest that trehalose, when fed to plant shoots grown *in vitro* is toxic or inhibitory to the growth of plant cells (Veluthambi K. et al., 1981, Plant Physiol. 68, 1369-1374). Plant cells producing low trehalase levels were found to be generally more sensitive to the adverse effects of trehalose, than plants exhibiting a higher level of trehalase activity. Trehalose-analogs, such as trehalose-amines were used to inhibit trehalase activity in shoots, making it possible to study the effects of trehalose fed to plant cells. Plant shoots which produce relatively high amounts of trehalase were adversely affected by the addition of trehalase inhibitors. Inhibition of trehalase activity in homogenates of callus and suspension culture of various *Angiospermae* using Validamycin is disclosed by Kendall et al., 1990, Phytochemistry 29, 2525-2582.

It is an object of the present invention to provide plants and plant parts capable of producing and accumulating trehalose, while keeping any adverse effects that may arise from the accumulation of trehalose within acceptable limits.

SUMMARY OF THE INVENTION

The invention provides a process for producing trehalose in plant cells capable of producing trehalase by growing plant cells having the genetic information required for the production of trehalose and trehalase, or cultivating a plant or a part thereof comprising such plant cells, characterised in that said plant cells are grown, or said plant or a part thereof, is cultivated in the presence of a trehalase inhibitor. Preferred plants or plant parts or plant cells have been genetically altered so as to contain a chimeric trehalose phosphate synthase gene in a plant expressible form. According to one embodiment said trehalose phosphate synthase gene comprises an open reading frame encoding trehalose phosphate synthase from *E. coli* in plant expressible form.

According to a further aspect of the invention, plants have been genetically altered so as to produce trehalose preferentially in certain tissues or parts, such as (micro-)tubers of potato. According to one embodiment the open reading frame encoding trehalose phosphate synthase from *E. coli* is downstream of the potato patatin promoter, to provide for preferential expression of the gene in tubers and micro-tubers of *Solanum tuberosum*.

According to another aspect of the invention the plants are cultivated *in vitro*, for example in hydroculture.

10 According to another preferred embodiment said trehalase inhibitor comprises validamycin A in a form suitable for uptake by said plant cells, preferably in a concentration between 100 nM and 10 mM, preferably between 0.1 and 1 mM, in aqueous solution.

Equally suitable said trehalase inhibition can be formed by transformation of said plant with the antisense gene to the gene encoding the information for trehalase.

Also suitable as trehalase inhibitor is the 86 kD protein from the american cockroach (*Periplaneta americana*). This protein can be administered to a plant in a form suitable for uptake, and also it is possible that the plants are transformed with DNA coding for said protein.

The invention further provides plants and plant parts which accumulate trehalose in an amount above 0.01 % (fresh weight), preferably of a *Solanaceae* species, in particular *Solanum tuberosum* or *Nicotiana tabacum*, in particular a micro-tuber of *Solanum tuberosum* containing trehalose.

The invention also comprises the use of a plant, or plant part, according to the invention for extracting trehalose, as well as the use thereof in a process of forced extraction of water from said plant or plant part. According to yet another embodiment of the invention a chimaeric plant expressible gene is provided, comprising in sequence a transcription initiation region obtainable from a gene, preferentially expressed in a plant part, particularly the patatin gene from *Solanum tuberosum*, a 5'-untranslated leader, an open reading frame encoding a trehalose phosphate synthase activity, and downstream of said open reading frame a transcriptional terminator region.

According to yet another embodiment of the invention a chimaeric plant expressible gene is provided, comprising in sequence a transcription initiation region obtainable from a gene, preferentially expressed in a plant part, particularly the patatin gene from *Solanum tuberosum*, a 5'-untranslated leader, an open reading frame encoding a trehalase coupled in the antisense orientation, and downstream of said open reading frame a transcriptional terminator region. A preferred plant expressible gene according to the invention is one wherein said transcriptional terminator region is obtainable from the proteinase inhibitor-II gene of *Solanum tuberosum*. The invention also provided vectors and recombinant plant genomes comprising a chimaeric plant expressible gene according to the invention, as well as a plant cell having a recombinant genome, a plant or a part thereof, consisting essentially of cells. A further preferred plant species according to this aspect is *Solanum tuberosum*, and a micro-tuber thereof.

The invention further provides a process for obtaining trehalose, comprising the steps of growing plant cells according to the invention or cultivating a plant according to the invention and extracting trehalose from said plant cells, plants or parts.

The following figures further illustrate the invention.

DESCRIPTION OF THE FIGURES

Figure 1. Schematic representation of binary vector pMOG799.

Figure 2. Schematic representation of binary vector pMOG845.

Figure 3. Schematic representation of parts of the sucrose and starch biosynthetic pathways in plant sink tissues. The figure shows that carbohydrate produced in the leaf by photosynthesis is transported via the phloem tissue in the form of sucrose. Upon entering the sink it is unloaded by a membrane bound invertase activity to yield the monosugars glucose and fructose. By the action of a number of enzymatic steps these monosugars are converted to starch and/or sucrose as roughly shown here. The glucose metabolites G6P and UDPG are believed to be used as the substrates for the TPS-enzyme engineered into the plant by introduction of the plant expressible *otsA* gene. The figure shows how the amount of UDPG and G6P available as substrate is increased by reducing the levels of the enzymes SPS and AGPase. Their inhibition is marked with a cross.

Figure 4. Alignments for maximal amino acid similarities of neutral trehalase from *S. cerevisiae* with periplasmatic trehalase from *E. coli*, small intestinal trehalases from rabbit and trehalase from pupal midgut of the silkworm, *Bombyx mori*. Identical residues among all trehalase enzymes are indicated in **bold italics** typeface. Conserved regions of the amino acid sequences were aligned to give the best fit. Gap's in the amino acid sequence are represented by dashes.

Positions of degenerated primers based on conserved amino acids are indicated by dashed arrows.

DETAILED DESCRIPTION OF THE INVENTION

According to the present invention it has been found that the accumulation of an increased level of trehalose in plants and plant parts is feasible, without causing too drastic effects on the viability of the plant or plant parts. This important finding can be exploited by adapting plant systems to produce and/or accumulate high levels of trehalose at lower cost.

According to one embodiment of the invention the accumulation of increased levels of trehalose is achieved by inhibiting endogenous trehalases. Inhibition of trehalases can be performed basically in two ways: by administration of trehalase inhibitors exogenously, and by the production of trehalase inhibitors endogenously, for instance by transforming the plants with DNA sequences coding for trehalase inhibitors.

This inhibition can be equally well applied to plants which are transformed with enzymes which enable the production of trehalose, but also to plants which are able to synthesize trehalose naturally.

According to this first embodiment of the invention, trehalase inhibitors are administered to the plant system exogenously. Examples of trehalase inhibitors that may be used in such a process according to the invention are trehazolin produced in *Micromonospora*, strain SANK 62390 (Ando et al., 1991, J. Antibiot. **44**, 1165-1168), validoxylamine A, B, G, D-glucosyl-Dihydrovalidoxylamine A, L-ido-Dihydrovalidoxylamine A, Deoxynojirimycin (Kameda et al., 1987, J. Antibiot. **40**(4), 563-565), 5-epi-trehazolin (Trehalostatin) (Kobayashi Y. et al., 1994, J. Antibiot. **47**, 932-938), castanospermin (Salleh H.M. & Honek J.F. March 1990, FEBS **262**(2), 359-362) and the 86kD protein from the american cockroach (*Periplaneta*

americana) (Hayakawa et al., 1989, J. Biol. Chem. 264(27), 16165-16169).

A preferred trehalase inhibitor according to the invention is validamycin A (1,5,6-trideoxy-3-O- β -D-glucopyranosyl-5-(hydroxymethyl)-1-[[4,5,6-trihydroxy-3-(hydroxymethyl)-2-cyclohexen-1-yl]amino]-D-chiro-inositol).

5 Trehalase inhibitors are administered to plants or plant parts, or plant cell cultures, in a form suitable for uptake by the plants, plant parts or cultures. Typically the trehalase inhibitor is in the form of an aqueous solution of between 100 nM and 10 mM of active ingredient, preferably between 0.1 and 1 mM. Aqueous solutions may be applied to plants or plant
10 parts by spraying on leaves, watering, adding it to the medium of a hydroculture, and the like. Another suitable formulation of validamycin is solacol, a commercially available agricultural formulation (Takeda Chem. Indust., Tokyo).

Alternatively, or in addition to using exogenously administered
15 trehalase inhibitors, trehalase inhibitors may be provided by introducing the genetic information coding therefor. One form of such in-built trehalase inhibitor may consist of a genetic construct causing the production of RNA that is sufficiently complementary to endogenous RNA encoding for trehalase to interact with said endogenous transcript, thereby
20 inhibiting the expression of said transcript. This so-called "antisense approach" is well known in the art (*vide inter alia* EP 0 240 208 A and the Examples to inhibit SPS disclosed in WO 95/01446).

A gene coding for trehalase has been isolated from a potato cDNA library and sequenced. The predicted amino acid sequence of trehalase as
25 shown in SEQIDNO:10 is derived from the nucleotide sequence depicted in SEQIDNO: 9. As is well known in the biological arts amino acid sequences of equivalent enzymes can differ between species. It is emphasized that the difference between the known trehalase sequences and plant trehalase sequence makes it very questionable if such trehalase sequence used in an
30 antisense approach is capable of inhibiting trehalase expression *in planta*.

Of course the most preferred embodiment of the invention is obtained by transforming a plant with the antisense trehalase gene which matches exactly with the endogenous trehalase gene. However, sequences which have a high degree of homology can also be used. Thus, the antisense
35 trehalase gene to be used for the transformation of potato will be directed against the nucleotide sequence depicted in SEQIDNO: 9.

It is usually enough to express only part of the homologous gene in the antisense orientation, in order to achieve effective inhibition of expression of the endogenous trehalase (*vide* Van der Krol et al., 1990, Plant Molecular Biology, 14, 457-466).

5 Trehalase gene sequences of other plants can be elucidated in two different ways. One of the strategies is to use the isolated potato cDNA clone as a probe to screen a cDNA library containing the cDNA of the desired plant species. Positive reacting clones can then be isolated and subcloned into suitable vectors.

10 A second strategy to identify such genes is by purifying the proteins which are involved in trehalose degradation. An example for such a strategy is the purification of a protein with acid invertase activity from potato (*Solanum tuberosum* L.) tubers (Burch et al., Phytochemistry, Vol.31, No.6, pp. 1901-1904, 1992). The obtained protein
15 preparation also exhibits trehalose hydrolysing activity. Disaccharide hydrolysing activity of protein preparations obtained after purification steps can be monitored as described by Dahlqvist (Analytical Biochemistry 7, 18-25, 1964).

 After purifying the protein(s) with trehalose hydrolysing
20 activity to homogeneity, the N-terminal amino acid sequence or the sequence of internal fragments after protein digestion is determined. These sequences enable the design of oligonucleotide probes which are used in a polymerase chain reaction (PCR) or hybridization experiments to isolate the corresponding mRNAs using standard molecular cloning techniques.

25 An isolated cDNA encoding a trehalose degrading enzyme is subsequently fused to a promoter sequence in such a way that transcription results in the synthesis of antisense mRNA.

 Another form of such an in-built trehalase inhibitor may consist of a genetic construct causing the production of a protein that is able to
30 inhibit trehalase activity in plants. A proteinaceous inhibitor of trehalase has been isolated and purified from the serum of resting adult american cockroaches (*Periplaneta americana*) (Hayakawa et al., *supra*). This protein, of which the sequence partly has been described in said publication, can be made expressable by isolation of the gene coding for
35 the protein, fusion of the gene to a suitable promoter, and transformation of said fused gene into the plant according to standard molecular

biological methods.

A promoter may be selected from any gene capable of driving transcription in plant cells.

If trehalose accumulation is only desired in certain plant parts, such as potato (mini-)tubers, the trehalase inhibitory DNA construct (e.g. the antisense construct) comprises a promoter fragment that is preferentially expressed in (mini-)tubers, allowing endogenous trehalase levels in the remainder of the plant's cells to be substantially unaffected. Thus, any negative effects of trehalose to neighbouring plant cells due to trehalose diffusion, is counteracted by unaffected endogenous trehalase activity in the remainder of the plant.

In the Example illustrating the invention, wherein trehalose phosphate synthase is produced under the control of the patatin promoter fragment, also the trehalase-inhibitory construct may comprise a promoter fragment of the patatin gene.

Mutatis mutandis if trehalose is to be accumulated in tomato fruit, both a plant expressible trehalose phosphate synthase gene, which is at least expressed in the tomato fruit is to be used, as well as a plant expressible trehalase-inhibitory DNA construct, which should be expressed preferentially in the fruit, and preferably not, or not substantially, outside the fruit. An example of a promoter fragment that may be used to drive expression of DNA-constructs preferentially in tomato fruit is disclosed in EP 0 409 629 A1. Numerous modifications of this aspect of the invention, that do not depart from the scope of this invention, are readily envisaged by persons having ordinary skill in the art to which this invention pertains.

An alternative method to block the synthesis of undesired enzymatic activity such as caused by endogenous trehalase is the introduction into the genome of the plant host of an additional copy of said endogenous trehalase gene. It is often observed that the presence of a transgene copy of an endogenous gene silences the expression of both the endogenous gene and the transgene (EP 0 465 572 A1).

According to one embodiment of the invention accumulation of trehalose is brought about in plants wherein the capacity of producing trehalose has been introduced by introduction of a plant expressible gene construct encoding trehalose phosphate synthase (TPS).

Any trehalose phosphate synthase gene under the control of regulatory elements necessary for expression of DNA in plant cells, either specifically or constitutively, may be used, as long as it is capable of producing an active trehalose phosphate synthase activity. A preferred open
5 reading frame according to the invention is one encoding a TPS-enzyme as represented in SEQIDNO: 2. It is well known that more than one DNA sequence may encode an identical enzyme, which fact is caused by the degeneracy of the genetic code. If desired, the open reading frame encoding the trehalose phosphate synthase activity may be adapted to codon usage in the host plant
10 of choice, but this is not a requirement.

The isolated nucleic acid sequence represented by SEQIDNO: 2, may be used to identify trehalose phosphate synthase activities in other organisms and subsequently isolating and cloning them, by hybridizing DNA from other sources with a DNA- or RNA fragment obtainable from the *E. coli*
15 gene. Preferably, such DNA sequences are screened by hybridizing under more or less stringent conditions (such as temperature and ionic strength of the hybridization mixture). Whether or not conditions are stringent also depends on the nature of the hybridization, *i.e.* DNA:DNA, DNA:RNA, RNA:RNA, as well as the length of the shortest hybridizing fragment. Those of skill
20 in the art are readily capable of establishing a hybridization regime stringent enough to isolate TPS genes, while avoiding aspecific hybridization. As genes involved in trehalose synthesis from other sources become available these can be used in a similar way to obtain a plant expressible trehalose phosphate synthase gene according to the invention.

25 Sources for isolating trehalose phosphate synthase activities include microorganisms (*e.g.* bacteria, yeast, fungi), plants, animals, and the like. Isolated DNA sequences encoding trehalose phosphate activity from other sources may be used likewise in a method for producing trehalose according to the invention. As an example, genes for producing trehalose
30 from yeast are disclosed in WO 93/17093.

The invention also encompasses nucleic acid sequences which have been obtained by modifying the nucleic acid sequence represented in SEQIDNO: 2 by mutating one or more codons so that it results in amino acid changes in the encoded protein, as long as mutation of the amino acid
35 sequence does not entirely abolish trehalose phosphate synthase activity.

According to another embodiment of the invention, plants are

genetically altered to produce and accumulate trehalose in specific parts of the plant, which were selected on the basis of considerations such as substrate availability for the TPS-enzyme, insensitivity of the plant part to any putative adverse effects of trehalose on plant cell functioning, and the like. A preferred site of TPS- enzyme expression are starch storage parts of plants. In particular microtubers potato are considered to be suitable plant parts. A preferred promoter to achieve selective TPS-enzyme expression in microtubers and tubers of potato is obtainable from the region upstream of the open reading frame of the patatin gene of potato (*Solanum tuberosum*).

Plants may be further modified by introducing additional genes that encode phosphatases that are capable of the conversion of trehalose phosphate into trehalose. At least in potato tubers or micro-tubers, potato leaves and tobacco leaves and roots, endogenous phosphatase activity appears to be present, so that the introduction of a trehalose phosphate phosphatase (TPP) gene is not an absolute requirement.

According to another embodiment of the invention, trehalose accumulation is further enhanced by the inhibition of endogenous genes in order to enhance substrate availability for the trehalose phosphate synthase, as exemplified herein with the inhibition of endogenous sucrose phosphate synthase gene and the ADP-Glucose pyrophosphorylase gene (AGP-ase). Inhibition of undesired endogenous enzyme activity is achieved in a number of ways, the choice of which is not critical to the invention. Preferably gene inhibition is achieved through the so-called 'antisense approach'. Herein a DNA sequence is expressed which produces an RNA that is at least partially complementary to the RNA which encodes the enzymatic activity that is to be blocked (e.g. AGP-ase or SPS (Sucrose Phosphate Synthase), in the examples). It is preferred to use homologous antisense genes as these are more efficient than heterologous genes. The isolation of an antisense SPS gene from potato using a maize SPS-gene sequence as probe serves to illustrate the feasibility of this strategy. It is not meant to indicate that, for practicing the invention the use of homologous antisense fragments is required. An alternative method to block the synthesis of undesired enzymatic activities is the introduction into the genome of the plant host of an additional copy of an endogenous gene present in the plant host. It is often observed that such an additional copy of a gene silences

the endogenous gene: this effect is referred to in the literature as the co-suppressive effect, or co-suppression. Details of the procedure of enhancing substrate availability are provided in the Examples of WO 95/01446, incorporated by reference herein.

5 Preferred plant hosts among the *spermatophyta* are the *Angiospermae*, notably the *Dicotyledoneae*, comprising *inter alia* the *Solanaceae* as a representative family, and the *Monocotyledoneae*, comprising *inter alia* the *Gramineae* as a representative family. Suitable host plants, as defined in the context of the present invention include plants (as well
10 as parts and cells of said plants) and their progeny which have been genetically modified using recombinant DNA techniques to cause or enhance production of trehalose in the desired plant or plant organ; these plants may be used directly (e.g. the plant species which produce edible parts) in processing or the trehalose may be extracted and/or purified from said
15 host. Crops with edible parts according to the invention include those which have flowers such as cauliflower (*Brassica oleracea*), artichoke (*Cynara scolymus*), fruits such as apple (*Malus*, e.g. *domesticus*), banana (*Musa*, e.g. *acuminata*), berries (such as the currant, *Ribes*, e.g. *rubrum*), cherries (such as the sweet cherry, *Prunus*, e.g. *avium*), cucumber (*Cucumis*,
20 e.g. *sativus*), grape (*Vitis*, e.g. *vinifera*), lemon (*Citrus limon*), melon (*Cucumis melo*), nuts (such as the walnut, *Juglans*, e.g. *regia*; peanut, *Arachis hypogaeae*), orange (*Citrus*, e.g. *maxima*), peach (*Prunus*, e.g. *persica*), pear (*Pyra*, e.g. *communis*), pepper (*Solanum*, e.g. *capsicum*), plum (*Prunus*, e.g. *domestica*), strawberry (*Fragaria*, e.g. *moschata*), tomato
25 (*Lycopersicon*, e.g. *esculentum*), leafs, such as alfalfa (*Medicago sativa*), cabbages (such as *Brassica oleracea*), endive (*Cichoreum*, e.g. *endivia*), leek (*Allium porrum*), lettuce (*Lactuca sativa*), spinach (*Spinaciaoleraceae*), tobacco (*Nicotiana tabacum*), roots, such as arrowroot (*Maranta arundinacea*), beet (*Beta vulgaris*), carrot (*Daucus carota*),
30 cassava (*Manihot esculenta*), turnip (*Brassica rapa*), radish (*Raphanus sativus*), yam (*Dioscorea esculenta*), sweet potato (*Ipomoea batatas*) and seeds, such as bean (*Phaseolus vulgaris*), pea (*Pisum sativum*), soybean (*Glycin max*), wheat (*Triticum aestivum*), barley (*Hordeum vulgare*), corn (*Zea mays*), rice (*Oryza sativa*), tubers, such as kohlrabi (*Brassica oleraceae*), potato (*Solanum tuberosum*), and the like. The edible parts may
35 be conserved by drying in the presence of enhanced trehalose levels

produced therein due to the presence of a plant expressible trehalose phosphate synthase gene.

The method of introducing the plant expressible trehalose-phosphate synthase gene, or any other sense or antisense gene into a recipient plant cell is not crucial, as long as the gene is expressed in said plant cell. The use of *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* - mediated transformation is preferred, but other procedures are available for the introduction of DNA into plant cells. Examples are transformation of protoplasts using the calcium/polyethylene glycol method, electroporation microinjection and DNA-coated particle bombardment (Potrykus, 1990, Bio/Technol. 8, 535-542). Also combinations of *Agrobacterium* and coated particle bombardment may be used. Also transformation protocols involving other living vectors than *Agrobacterium* may be used, such as viral vectors (e.g. from the Cauliflower Mosaic Virus (CaMV) and or combinations of *Agrobacterium* and viral vectors, a procedure referred to as agroinfection (Grimsley N. et al., 8 January 1987, Nature 325, 177-179). After selection and/or screening, the protoplasts, cells or plant parts that have been transformed are regenerated into whole plants, using methods known in the art (Horsch et al., 1985, Science 225, 1229-1231).

The development of reproducible tissue culture systems for monocotyledonous crops, together with methods for introduction of genetic material into plant cells has facilitated transformation. Presently, preferred methods for transformation of monocot species are microprojectile bombardment of explants or suspension cells, and direct DNA uptake or electroporation (Shimamoto, et al., 1989, Nature 338, 274-276). Transgenic maize plants have been obtained by introducing the *Streptomyces hygroscopicus* bar-gene, which encodes phosphinothricin acetyltransferase (an enzyme which inactivates the herbicide phosphinothricin), into embryogenic cells of a maize suspension culture by microprojectile bombardment (Gordon-Kamm, 1990, Plant Cell, 2, 603-618). The introduction of genetic material into aleurone protoplasts of other monocot crops such as wheat and barley has been reported (Lee, 1989, Plant Mol. Biol. 13, 21-30). Wheat plants have been regenerated from embryogenic suspension culture by selecting only the aged compact and nodular embryogenic callus tissues for the establishment of the embryogenic suspension cultures

(Vasil, 1990 Bio/Technol. 8, 429-434).

Monocotyledonous plants, including commercially important crops such as corn and rice may be obtained by *Agrobacterium*-mediated transformation according to Gould J, Michael D, Hasegawa O, Ulian EC, Peterson G, Smith RH, (1991) Plant. Physiol. 95, 426-434; Hiei Y. et al., The Plant Journal 6(2), 271-282 and European patent 159 418 B1.

Suitable DNA sequences for control of expression of the plant expressible genes (including marker genes), such as transcriptional initiation regions, enhancers, non-transcribed leaders and the like, may be derived from any gene that is expressed in a plant cell. Also intended are hybrid promoters combining functional portions of various promoters, or synthetic equivalents thereof. Apart from constitutive promoters, inducible promoters, or promoters otherwise regulated in their expression pattern, e.g. developmentally or cell-type specific, may be used to control expression of the plant expressible genes according to the invention as long as they are expressed in plant parts that contain substrate for TPS.

To select or screen for transformed cells, it is preferred to include a marker gene linked to the plant expressible gene according to the invention to be transferred to a plant cell. The choice of a suitable marker gene in plant transformation is well within the scope of the average skilled worker; some examples of routinely used marker genes are the neomycin phosphotransferase genes conferring resistance to kanamycin (EP-B 131 623), the Glutathion-S-transferase gene from rat liver conferring resistance to glutathione derived herbicides (EP-A 256 223), glutamine synthetase conferring upon overexpression resistance to glutamine synthetase inhibitors such as phosphinothricin (WO87/05327), the acetyl transferase gene from *Streptomyces viridochromogenes* conferring resistance to the selective agent phosphinothricin (EP-A 275 957), the gene encoding a 5-enolshikimate-3- phosphate synthase (EPSPS) conferring tolerance to N-phosphonomethylglycine, the *bar* gene conferring resistance against Bialaphos (e.g. WO 91/02071) and the like. The actual choice of the marker is not crucial as long as it is functional (i.e. selective) in combination with the plant cells of choice.

The marker gene and the gene of interest do not have to be linked, since co-transformation of unlinked genes (U.S. Patent 4,399,216) is also an efficient process in plant transformation.

Preferred plant material for transformation, especially for dicotyledonous crops are leaf-discs which can be readily transformed and have good regenerative capability (Horsch R.B. et al., (1985) Science 227, 1229-1231).

5 It is immaterial to the invention how the presence of two or more genes in the same plant is effected. This can *inter alia* done be achieved by one of the following methods:

- (a) transformation of the plant line with a multigene construct containing more than one gene to be introduced,
- 10 (b) co-transforming different constructs to the same plant line simultaneously,
- (c) subsequent rounds of transformation of the same plant with the genes to be introduced,
- (d) crossing two plants each of which contains a different gene to be
- 15 introduced into the same plant, or
- (e) combinations thereof.

The field of application of the invention lies both in agriculture and horticulture, for instance due to improved properties of the modified plants as such (e.g. stress tolerance, such as cold tolerance,

20 and preferably drought resistance, and increase in post-harvest quality and shelf-life of plants and plant products), as well as in any form of industry where trehalose is or will be applied in a process of forced water extraction, such as drying or freeze drying. Trehalose can be used or sold as such, for instance in purified form or in admixtures, or in the form of

25 a plant product, such as tuber, a fruit, a flower containing the trehalose, either in native state or in (partially) dehydrated form, and the like. Plant parts harbouring (increased levels of) trehalose phosphate or trehalose may be used or sold as such or processed without the need to add trehalose.

30 Also trehalose can be extracted and/or purified from the plants or plant parts producing it and subsequently used in an industrial process. In the food industries trehalose can be employed by adding trehalose to foods before drying. Drying of foods is an important method of preservation. Trehalose seems especially useful to conserve food products

35 through conventional air-drying, and to allow for fast reconstitution upon addition of water of a high quality product (Roser et al., July 1991,

Trends in Food Science and Technology, pp. 166-169). The benefits include retention of natural flavors/fragrances, taste of fresh product, and nutritional value (proteins and vitamins). It has been shown that trehalose has the ability to stabilize proteins e.g. vaccines, enzymes and membranes, and to form a chemically inert, stable glass. The low water activity of such thoroughly dried food products prevents chemical reactions, that could cause spoilage.

Field crops like corn, cassava, potato, sugar beet and sugarcane have since long been used as a natural source for bulk carbohydrate production (starches and sucrose). The production of trehalose in such crops, facilitated by genetic engineering of the trehalose-biosynthetic pathway into these plant species, would allow the exploitation of such engineered crops for trehalose production.

Trehalose is also used in drying or storage of biological macromolecules, such as peptides, enzymes, polynucleotides and the like.

All references cited in this specification are indicative of the level of skill in the art to which the invention pertains. All publications, whether patents or otherwise, referred to previously or later in this specification are herein incorporated by reference as if each of them was individually incorporated by reference. In particular WO 95/01446, cited herein, describing the production of trehalose in higher plants by genetic manipulation is herein incorporated by reference.

The Examples given below illustrate the invention and are in no way intended to indicate the limits of the scope of the invention.

Experimental

DNA manipulations

All DNA procedures (DNA isolation from *E.coli*, restriction, ligation, transformation, etc.) are performed according to standard protocols (Sambrook et al. (1989) Molecular Cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, CSH, New York).

Strains

In all examples *E.coli* K-12 strain DH5 α is used for cloning. The *Agrobacterium tumefaciens* strains used for plant transformation experiments

are EHA 105 and MOG 101 (Hood et al. 1993, Trans. Research 2, 208-218)

Construction of Agrobacterium strain MOG101

A binary vector system (Hoekema A., Hirsch, P.R., Hooykaas, P.J.J., and Schilperoort, R.A. (1983) Nature 303, 179) is used to transfer gene constructs into potato and tobacco plants. The helper plasmid conferring the *Agrobacterium tumefaciens* virulence functions is derived from the octopine Ti-plasmid pTiB6. MOG101 is an *Agrobacterium tumefaciens* strain carrying a non-oncogenic Ti-plasmid (Koekman et al. 1982, supra) from which the entire T-region is deleted and substituted by a bacterial Spectinomycin resistance marker from transposon Tn1831 (Hooykaas et al., 1980 Plasmid 4, 64-75). The Ti-plasmid pTiB6 contains two adjacent T-regions, TL (T-left) and TR (T-right). To obtain a derivative lacking the TL- and TR-regions, we constructed intermediate vector pMOG579. Plasmid pMOG579 is a pBR322 derivative which contains 2 Ti-plasmid fragments homologous to the fragments located left and right outside the T-regions of pTiB6. The 2 fragments are separated in pMOG579 by a 2.5 kb BamHI - HindIII fragment from transposon Tn1831 (Hooykaas et al., 1980 Plasmid 4, 64-75) carrying the spectinomycin resistance marker. The plasmid is introduced into *Agrobacterium tumefaciens* strain LBA1010 [C58-C9 (pTiB6) = a cured C58 strain in which pTiB6 is introduced (Koekman et al. (1982), supra), by triparental mating from *E.coli*, using HB101 8pRK2013 as a helper. Transconjugants are selected for resistance to Rifampicin (20 mg/l) and spectinomycin (250 mg/l). A double recombination between pMOG579 and pTiB6 resulted in loss of carbenicillin resistance (the pBR322 marker) and deletion of the entire T-region. Of 5000 spectinomycin resistant transconjugants replica plated onto carbenicillin (100 mg/l) 2 are found sensitive. Southern analysis (not shown) showed that a double crossing over event had deleted the entire T-region. The resulting strain is called MOG101. This strain and its construction is analogous to strain GV2260 (Deblaere et al. 1985, Nucl. Acid Res. 13, 4777-4788).

An alternative helper strain for MOG101 is e.g. LBA4404; this strain can also suitably be used for introduction of a binary plasmid, such as pMOG799 and subsequent plant transformation. Other suitable helper strains are readily available.

Isolation of a patatin promoter/construction of pMOG546

A patatin promoter fragment is isolated from chromosomal DNA of *Solanum tuberosum* cv. Bintje using the polymerase chain reaction. A set of oligonucleotides, complementary to the sequence of the upstream region of the λ pat21 patatin gene (Bevan, M., Barker, R., Goldsbrough, A., Jarvis, M., Kavanagh, T. and Iturriaga, G. (1986) *Nucleic Acids Res.* **14**: 5564-5566), is synthesized consisting of the following sequences:

```

5' AAG CTT ATG TTG CCA TAT AGA GTA G 3'   PatB33.2 (SEQIDNO:3)
5' GTA GTT GCC ATG GTG CAA ATG TTC 3'   PatATG.2 (SEQIDNO:4)

```

These primers are used to PCR amplify a DNA fragment of 1123bp, using chromosomal DNA isolated from potato cv. Bintje as a template. The amplified fragment shows a high degree of similarity to the λ pat21 patatin sequence and is cloned using EcoRI linkers into a pUC18 vector resulting in plasmid pMOG546.

Construction of pMOG 799

pMOG 799 harbours the TPS gene from *E. coli* under control of the double enhanced 35S Cauliflower Mosaic promoter. The construction of this binary vector is described in detail in International patent application PCT/EP94/02167, incorporated herein by reference. A sample of an *E. coli* strain harbouring pMOG799 has been deposited under the Budapest Treaty at the Centraal Bureau voor Schimmelcultures, Oosterstraat 1, P.O. Box 273, 3740 AG Baarn, The Netherlands, on Monday 23 August, 1993: the Accession Number given by the International Depositary Institution is CBS 430.93.

Construction of pMOG845.

Plasmid pMOG546 containing the patatin promoter is digested with NcoI-KpnI, incubated with *E. coli* DNA polymerase I in the presence of dATP and dCTP thereby destroying the NcoI and KpnI site and subsequently religated. From the resulting vector a 1.1kb EcoRI-SmaI fragment containing the patatin promoter is isolated and cloned into pMOG798 (described in detail in PCT/EP94/02167) linearized with SmaI-EcoRI consequently exchanging the 35S CaMV promoter for the patatin promoter. The resulting vector is linearized with HindIII and ligated with the following oligonucleotide duplex:

	(HindIII)	PstI	KpnI	HindIII	
	5'	AGCT CTGCAG TGA GGTACC A	3'	TCV 11 (SEQIDNO:5)	
5	3'	GACGTC ACT CCATGG TTCGA	5'	TCV 12 (SEQIDNO:6)	

After checking the orientation of the introduced oligonucleotide duplex, the resulting vector is linearized with PstI-HindIII followed by the insertion of a 950bp PstI-HindIII fragment harbouring the potato proteinase inhibitor II terminator (PotPiII) (An, G., Mitra, A., Choi, H.K., Costa, M.A., An, K., Thornburg, R. W. and Ryan, C.A. (1989) The Plant Cell 1: 115-122). The PotPiII terminator is isolated by PCR amplification using chromosomal DNA isolated from potato cv. Desiree as a template and the following set of oligonucleotides:

15	5'	GTACCCTGCAGTGTGACCCTAGAC	3'	TCV 15 (SEQIDNO:7)
	5'	TCGATTCATAGAAGCTTAGAT	3'	TCV 16 (SEQIDNO:8)

The TPS expression cassette is subsequently cloned as a EcoRI-HindIII fragment into the binary vector pMOG402 resulting in pMOG845 (fig. 4). A sample of *E.coli* Dh α strain, harbouring pMOG845 has been deposited at the Centraal Bureau voor Schimmelcultures, Oosterstraat 1, P.O. Box 273, 3740 AG Baarn, The Netherlands, on January 4, 1995; the Accession Number given by the International Depositary Institution is CBS 101.95.

25

Triparental matings

The binary vectors are mobilized in triparental matings with the *E. coli* strain HB101 containing plasmid pRK2013 (Ditta G., Stanfield, S., Corbin, D., and Helinski, D.R. et al. (1980) Proc. Natl. Acad. Sci. USA 77, 7347) into *Agrobacterium tumefaciens* strain MOG101 or EHA105 and used for transformation.

Transformation of tobacco (*Nicotiana tabacum* SR1)

Tobacco is transformed by cocultivation of plant tissue with *Agrobacterium tumefaciens* strain MOG101 containing the binary vector of interest as described. Transformation is carried out using cocultivation of tobacco

35

(*Nicotiana tabacum* SR1) leaf disks as described by Horsch et al. 1985, Science 227, 1229-1231. Transgenic plants are regenerated from shoots that grow on selection medium containing kanamycin, rooted and transferred to soil.

5

Transformation of potato

Potato (*Solanum tuberosum* cv. Kardal) is transformed with the *Agrobacterium* strain EHA 105 containing the binary vector of interest. The basic culture medium is MS30R3 medium consisting of MS salts (Murashige, T. and Skoog, F. 10 (1962) *Physiol. Plan.* 14, 473), R3 vitamins (Ooms et al. (1987) *Theor. Appl. Genet.* 73, 744), 30 g/l sucrose, 0.5 g/l MES with final pH 5.8 (adjusted with KOH) solidified when necessary with 8 g/l Daichin agar. Tubers of *Solanum tuberosum* cv. Kardal are peeled and surface sterilized by burning them in 96% ethanol for 5 seconds. Extinguish the flames in sterile 15 water and cut slices of approximately 2 mm thickness. Disks are cut with a bore from the vascular tissue and incubated for 20 minutes in MS30R3 medium containing 1-5 x10⁸ bacteria/ml of *Agrobacterium* EHA 105 containing the binary vector. Wash the tuber discs with MS30R3 medium and transfer them to solidified postculture medium (PM). PM consists of M30R3 medium 20 supplemented with 3.5 mg/l zeatin riboside and 0.03 mg/l indole acetic acid (IAA). After two days, discs were transferred to fresh PM medium with 200 mg/l cefotaxim and 100 mg/l vancomycin. Three days later, the tuber discs are transferred to shoot induction medium (SIM) which consists of PM medium with 250 mg/l carbenicillin and 100 mg/l kanamycin. After 4-8 weeks, shoots 25 emerging from the discs are excised and placed on rooting medium (MS30R3-medium with 100 mg/l cefotaxim, 50 mg/l vancomycin and 50 mg/l kanamycin). The shoots are propagated axenically by meristem cuttings.

Induction of micro-tubers

30 Stem segments of *in vitro* potato plants harbouring an auxiliary meristem are transferred to micro-tuber inducing medium. Micro-tuber inducing medium contains 1 X MS-salts supplemented with R3 vitamins, 0.5 g/l MES (final pH= 5.8, adjusted with KOH) and solidified with 8 g/l Daishin agar, 60 g/l sucrose and 2.5 mg/l kinetin. After 3 to 5 weeks of growth in the dark at 35 24°C, micro-tubers are formed.

Trehalose assay

Trehalose was determined quantitatively by anion exchange chromatography with pulsed amperometric detection. Extracts were prepared by adding 1 ml boiling water to 1 g frozen material which was subsequently heated for 15' at 100°C. Samples (25 µl) were analyzed on a Dionex DX-300 liquid chromatograph equipped with a 4 x 250 mm Dionex 35391 carbopac PA-1 column and a 4 x 50 mm Dionex 43096 carbopac PA-1 precolumn. Elution was with 100 mM NaOH at 1 ml/min. Sugars were detected with a pulsed amperometric detector (Dionex, PAD-2). Commercially available trehalose (Sigma) was used as a standard.

Isolation of Validamycin A

Validamycin A is isolated from Solacol, a commercial agricultural formulation (Takeda Chem. Indust., Tokyo) as described by Kendall et al. (1990) *Phytochemistry*, Vol. 29, No. 8, pp. 2525-2528. The procedure involves ion exchange chromatography (QAE-Sephadex A-25 (Pharmacia), bed vol. 10 ml, equilibration buffer 0.2 mM Na-Pi pH 7) from a 3% agricultural formulation of Solacol. Loading 1 ml of Solacol on the column and eluting with water in 7 fractions, practically all Validamycin is recovered in fraction 4.

Based on a 100% recovery, using this procedure, the concentration of Validamycin A was adjusted to 110^{-3} M in MS-buffer, for use in trehalose accumulation tests.

Alternatively, Validamycin A and B may be purified directly from *Streptomyces hygroscopicus* var. *limoneus*, as described by Iwasa T. et al., 1971, in *The Journal of Antibiotics* 24(2), 119-123, the content of which is incorporated herein by reference.

Construction of pMOG1027

pMOG1027 harbours the trehalase gene from *Solanum tuberosum* cv. Kardal in the reversed orientation under control of the double enhanced 35S Cauliflower Mosaic promoter. The construction of this vector is very similar to the construction of pMOG799 and can be performed by any person skilled in the art. After mobilization of this binary vector by triparental mating to *Agrobacterium*, this strain can be used to transform plant cells and to generate transgenic plants having reduced levels of trehalase

activity.

Construction of pMOG1028

pMOG1028 harbours the trehalase gene from *Solanum tuberosum* cv. Kardal in
 5 the reversed orientation under control of the tuber specific patatin
 promoter. The construction of this vector is very similar to the
 construction of pMOG845 and can be performed by any person skilled in the
 art. After mobilization of this binary vector by triparental mating to
Agrobacterium, this strain can be used in potato transformation experiments
 10 to generate transgenic plants having reduced levels of trehalase activity
 in tuber-tissue.

EXAMPLE 1

Cloning of a full length *E. coli* otsA gene

15 In *E. coli* trehalose phosphate synthase (TPS) is encoded by the
~~otsA~~ gene located in the operon ~~otsBA~~. The cloning and sequence
 determination of the otsA gene is described in detail in Example I of
 PCT/EP94/02167, herein incorporated by reference. To effectuate its
 expression in plant cells, the open reading frame has been linked to the
 20 transcriptional regulatory elements of the CaMV 35S RNA promoter, the
 translational enhancer of the ALMV leader, and the transcriptional
 terminator of the NOS-gene, as described in greater detail in Example I of
 PCT/EP94/02167.

A binary vector, pMOG799 (Fig. 1), containing the plant expressible otsA
 25 gene and a the kanamycin resistance gene as selectable marker between T-DNA
 borders, is used to transform potato and tobacco.

EXAMPLE 2

Trehalose production in tobacco plants transformed with pMOG799

30 Tobacco leaf discs are transformed with the binary vector pMOG799 using
Agrobacterium tumefaciens. Transgenic shoots are selected on kanamycin.
 Transgenic plants are transferred to the greenhouse to flower and set seed
 after selfing (S1). Seeds of these transgenic plants are surface sterilised
 and germinated *in vitro* on medium with Kanamycin. Kanamycin resistant
 35 seedlings and wild-type tobacco plants are transferred to MS-medium
 supplemented with 10^{-3} M Validamycin A. As a control, transgenic seedlings

and wild-type plants are transferred to medium without Validamycin A. Analysis of leaves and roots of plants grown on Validamycin A shows elevated levels of trehalose compared to the control plants (Table 1). No trehalose was detected in wild-type tobacco plants.

5

Table 1

	with Validamycin A		without Validamycin A	
	leaf	roots	leaf	roots
pMOG799.1	0.0081	0.0044	-	0.003
10 pMOG799.13	0.0110	0.0080	-	-
pMOG799.31	0.0008	0.0088	-	-
Wild-type SR1	-	-	-	-

EXAMPLE 315 Trehalose production in potato micro-tubers transformed with pMOG845

Potato *Solanum tuberosum* cv. Kardal tuber discs are transformed with *Agrobacterium tumefaciens* EHA105 harbouring the binary vector pMOG845. Transgenic shoots are selected on kanamycin. Micro-tubers (m-tubers) are induced on stem segments of transgenic and wild-type plants cultured on m-tuber inducing medium supplemented with 10^{-3} M Validamycin A. As a control, m-tubers are induced on medium without Validamycin A. M-tubers induced on medium with Validamycin A showed elevated levels of trehalose in comparison with m-tubers grown on medium without Validamycin A (Table 2). No trehalose was detected in wild-type m-tubers.

25

Table 2.

	Trehalose (% fresh weight)	
	+Validamycin A	-Validamycin A
845-2	0.016	-
30 845-4	-	-
845-8	0.051	-
845-13	0.005	-
845-22	0.121	-
845-25	0.002	-
35 wT Kardal	-	-

EXAMPLE 4

Trehalose production in hydrocultures of tobacco plants transformed with pMOG799

Seeds (S1) of selfed tobacco plants transformed with the binary vector
 5 pMOG799 are surface sterilised and germinated *in vitro* on MS20MS medium
 containing 50 µg/ml Kanamycin. Kanamycin resistant seedlings are
 transferred to soil and grown in a growth chamber (temp. 23°C, 16 hours of
 light/day). After four weeks, seedlings were transferred to hydrocultures
 with ASEF clay beads with approximately 450 ml of medium. The medium
 10 contains 40 g/l Solacol dissolved in nano-water buffered with 0.5 g/l MES
 to adjust to pH 6.0 which is sieved through a filter to remove solid
 particles. Essential salts are supplemented by adding POKONTM (1.5 ml/l).
 The following antibiotics are added to prevent growth of micro-organisms:
 500µg/ml Carbenicillin, 40µg/ml Nystatin and 100µg/ml Vancomycin. As a
 15 control, transgenic seedlings and wild-type plants are transferred to
 medium without Solacol. Analysis of leaves of plants grown on Solacol shows
 elevated levels of trehalose compared to the control plants (Table 3). No
 trehalose was detected in wild-type tobacco plants.

20 Table 3

	Solacol	Trehalose (%w/w)
pMOG 799.1-1	+	0.008
pMOG 799.1-2	+	0.004
pMOG 799.1-3	-	-
25 pMOG 799.1-4	-	-
pMOG 799.1-5	+	0.008
pMOG 799.1-6	-	-
pMOG 799.1-7	+	0.005
pMOG 799.1-8	-	-
30 pMOG 799.1-9	-	-
pMOG 799.1-10	+	0.007
Wild-type SR1-1	-	-
Wild-type SR1-2	+	-
35 Wild-type SR1-3	-	-
Wild-type SR1-4	+	-

Example 5

Cloning of a full length cDNA encoding trehalase from potato tuber tissue

Using the amino acid sequence of the conserved regions of known trehalase genes (*E.coli*, Yeast, Rabbit, *B. mori*) (figure 4), four degenerated primers were designed:

```

10      C   C   C   CGT   GT A   TTAT
      GG GGI G TT IGA T TA TGGGAC      Tase24 (SEQIDNO:11)
         T   A A TAA AG C   CGGC

15      GTICCGGIGGICGITT      Tase25 (SEQIDNO:12)
                        TAA   GT
                        CGT   AG

20      GGIGG T   GA   TG   A   A
          C   CT   ICGI CA IAG TA TA
                        G   G

25      C   G   AT   A
      I C TTI CCATCC AAICCTC
         G A   GC   G

```

Combinations of these primers in PCR experiments with genomic DNA and cDNA from *S. tuberosum* cv. Kardal leaf and tuber material respectively as template, resulted in several fragments of the expected length. A number of 190 bp. fragments obtained with the primer combination Tase24 and Tase 26 were subcloned into a pGEM T vector and sequenced. Several of the clones analyzed showed homology with known trehalase sequences. To exclude the isolation of non-plant derived trehalase sequences, Southern blot analysis was performed with gDNA from potato cv. Kardal. A number of clones isolated did not cross-hybridize with Kardal genomic DNA and were discarded. Two isolated clones were identical, gTase15.4 derived from a genomic PCR experiment and cTase5.2 derived from a PCR on cDNA, both showing hybridization in Southern blot analysis. One single hybridizing band was detected (EcoRI 1.5 Kb, HindIII 3 Kb and BamHI larger than 12 Kb) suggesting the presence of only one copy of the isolated PCR fragment.

A cDNA library was constructed out of poly A⁺ RNA from potato tubers (cv. Kardal) using a Stratagene cDNA synthesis kit and the vector Lambda ZAPII. Recombinant phages (500.000) were screened with the radiolabeled cTase5.2 PCR fragment resulting in the identification of 3 positive clones. After purification, two clones were characterised with restriction enzymes revealing inserts of 2.15 and 2.3 kb respectively. Their nucleotide sequence was 100% identical. The nucleic acid sequence

of on of these trehalase cDNA clones from *Solanum tuberosum* including its open reading frame is depicted in seq ID no:9, while the aminoacid sequence derived from this nucleic acid sequence is shown in seq. ID no:10. A plasmid harbouring an insert comprising the genetic information
5 coding for trehalase has been deposited under no. CBS 804.95 with the Centraal Bureau voor Schimmelcultures, Oosterstraat 1, P.O. Box 273, 3740 AG Baarn, the Netherlands on December 8, 1995.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- 5 (i) APPLICANT:
 (A) NAME: MOGEN International n.v.
 (B) STREET: Einsteinweg 97
 (C) CITY: LEIDEN
 (D) STATE: Zuid-Holland
10 (E) COUNTRY: The Netherlands
 (F) POSTAL CODE (ZIP): NL-2333 CB
 (G) TELEPHONE: (31).(71).5258282
 (H) TELEFAX: (31).(71).5221471
- 15 (ii) TITLE OF INVENTION: Enhanced accumulation of trehalose in plants
- (iii) NUMBER OF SEQUENCES: 14
- (iv) COMPUTER READABLE FORM:
20 (A) MEDIUM TYPE: Floppy disk
 (B) COMPUTER: IBM PC compatible
 (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 (D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)
- 25 (vi) PRIOR APPLICATION DATA:
 (A) APPLICATION NUMBER: EP 95.200.008.1
 (B) FILING DATE: 04-JAN-1995
- (vi) PRIOR APPLICATION DATA:
30 (A) APPLICATION NUMBER: EP 95.202.415.6
 (B) FILING DATE: 07-SEP-1995
- (2) INFORMATION FOR SEQ ID NO: 1:
- 35 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 1446 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: double
40 (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (iii) HYPOTHETICAL: NO
- 45 (iii) ANTI-SENSE: NO
- (vi) ORIGINAL SOURCE:
 (A) ORGANISM: Escherichia coli
50 (B) STRAIN: CLONE: 7F11
- (viii) POSITION IN GENOME:
 (B) MAP POSITION: 41-42'

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 19..1446

(D) OTHER INFORMATION: /product= "trehalose phosphate
synthase"
/gene= "otsA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

10	GAGAAAATAA CAGGAGTG ATG ACT ATG AGT CGT TTA GTC GTA GTA TCT AAC	51
	Met Thr Met Ser Arg Leu Val Val Val Ser Asn	
	1 5 10	
15	CGG ATT GCA CCA CCA GAC GAG CAC GCC GCC AGT GCC GGT GGC CTT GCC	99
	Arg Ile Ala Pro Pro Asp Glu His Ala Ala Ser Ala Gly Gly Leu Ala	
	15 20 25	
20	GTT GGC ATA CTG GGG GCA CTG AAA GCC GCA GGC GGA CTG TGG TTT GGC	147
	Val Gly Ile Leu Gly Ala Leu Lys Ala Ala Gly Gly Leu Trp Phe Gly	
	30 35 40	
25	TGG AGT GGT GAA ACA GGG AAT GAG GAT CAG CCG CTA AAA AAG GTG AAA	195
	Trp Ser Gly Glu Thr Gly Asn Glu Asp Gln Pro Leu Lys Lys Val Lys	
	45 50 55	
30	AAA GGT AAC ATT ACG TGG GCC TCT TTT AAC CTC AGC GAA CAG GAC CTT	243
	Lys Gly Asn Ile Thr Trp Ala Ser Phe Asn Leu Ser Glu Gln Asp Leu	
	60 65 70 75	
35	GAC GAA TAC TAC AAC CAA TTC TCC AAT GCC GTT CTC TGG CCC GCT TTT	291
	Asp Glu Tyr Tyr Asn Gln Phe Ser Asn Ala Val Leu Trp Pro Ala Phe	
	80 85 90	
40	CAT TAT CGG CTC GAT CTG GTG CAA TTT CAG CGT CCT GCC TGG GAC GGC	339
	His Tyr Arg Leu Asp Leu Val Gln Phe Gln Arg Pro Ala Trp Asp Gly	
	95 100 105	
45	TAT CTA CGC GTA AAT GCG TTG CTG GCA GAT AAA TTA CTG CCG CTG TTG	387
	Tyr Leu Arg Val Asn Ala Leu Leu Ala Asp Lys Leu Leu Pro Leu Leu	
	110 115 120	
50	CAA GAC GAT GAC ATT ATC TGG ATC CAC GAT TAT CAC CTG TTG CCA TTT	435
	Gln Asp Asp Asp Ile Ile Trp Ile His Asp Tyr His Leu Leu Pro Phe	
	125 130 135	
55	GCG CAT GAA TTA CGC AAA CGG GGA GTG AAT AAT CGC ATT GGT TTC TTT	483
	Ala His Glu Leu Arg Lys Arg Gly Val Asn Asn Arg Ile Gly Phe Phe	
	140 145 150 155	
60	CTG CAT ATT CCT TTC CCG ACA CCG GAA ATC TTC AAC GCG CTG CCG ACA	531
	Leu His Ile Pro Phe Pro Thr Pro Glu Ile Phe Asn Ala Leu Pro Thr	
	160 165 170	

	TAT GAC ACC TTG CTT GAA CAG CTT TGT GAT TAT GAT TTG CTG GGT TTC	579
	Tyr Asp Thr Leu Leu Glu Gln Leu Cys Asp Tyr Asp Leu Leu Gly Phe	
	175 180 185	
5	CAG ACA GAA AAC GAT CGT CTG GCG TTC CTG GAT TGT CTT TCT AAC CTG	627
	Gln Thr Glu Asn Asp Arg Leu Ala Phe Leu Asp Cys Leu Ser Asn Leu	
	190 195 200	
10	ACC CGC GTC ACG ACA CGT AGC GCA AAA AGC CAT ACA GCC TGG GGC AAA	675
	Thr Arg Val Thr Thr Arg Ser Ala Lys Ser His Thr Ala Trp Gly Lys	
	205 210 215	
15	GCA TTT CGA ACA GAA GTC TAC CCG ATC GGC ATT GAA CCG AAA GAA ATA	723
	Ala Phe Arg Thr Glu Val Tyr Pro Ile Gly Ile Glu Pro Lys Glu Ile	
	220 225 230 235	
20	GCC AAA CAG GCT GCC GGG CCA CTG CCG CCA AAA CTG GCG CAA CTT AAA	771
	Ala Lys Gln Ala Ala Gly Pro Leu Pro Pro Lys Leu Ala Gln Leu Lys	
	240 245 250	
25	GCG GAA CTG AAA AAC GTA CAA AAT ATC TTT TCT GTC GAA CGG CTG GAT	819
	Ala Glu Leu Lys Asn Val Gln Asn Ile Phe Ser Val Glu Arg Leu Asp	
	255 260 265	
30	TAT TCC AAA GGT TTG CCA GAG CGT TTT CTC GCC TAT GAA GCG TTG CTG	867
	Tyr Ser Lys Gly Leu Pro Glu Arg Phe Leu Ala Tyr Glu Ala Leu Leu	
	270 275 280	
35	GAA AAA TAT CCG CAG CAT CAT GGT AAA ATT CGT TAT ACC CAG ATT GCA	915
	Glu Lys Tyr Pro Gln His His Gly Lys Ile Arg Tyr Thr Gln Ile Ala	
	285 290 295	
40	CCA ACG TCG CGT GGT GAT GTG CAA GCC TAT CAG GAT ATT CGT CAT CAG	963
	Pro Thr Ser Arg Gly Asp Val Gln Ala Tyr Gln Asp Ile Arg His Gln	
	300 305 310 315	
45	CTC GAA AAT GAA GCT GGA CGA ATT AAT GGT AAA TAC GGG CAA TTA GGC	1011
	Leu Glu Asn Glu Ala Gly Arg Ile Asn Gly Lys Tyr Gly Gln Leu Gly	
	320 325 330	
50	TGG ACG CCG CTT TAT TAT TTG AAT CAG CAT TTT GAC CGT AAA TTA CTG	1059
	Trp Thr Pro Leu Tyr Tyr Leu Asn Gln His Phe Asp Arg Lys Leu Leu	
	335 340 345	
55	ATG AAA ATA TTC CGC TAC TCT GAC GTG GGC TTA GTG ACG CCA CTG CGT	1107
	Met Lys Ile Phe Arg Tyr Ser Asp Val Gly Leu Val Thr Pro Leu Arg	
	350 355 360	
60	GAC GGG ATG AAC CTG GTA GCA AAA GAG TAT GTT GCT GCT CAG GAC CCA	1155
	Asp Gly Met Asn Leu Val Ala Lys Glu Tyr Val Ala Ala Gln Asp Pro	
	365 370 375	

	GCC AAT CCG GGC GTT CTT GTT CTT TCG CAA TTT GCG GGA GCG GCA AAC	1203
	Ala Asn Pro Gly Val Leu Val Leu Ser Gln Phe Ala Gly Ala Ala Asn	
	380 385 390 395	
5	GAG TTA ACG TCG GCG TTA ATT GTT AAC CCC TAC GAT CGT GAC GAA GTT	1251
	Glu Leu Thr Ser Ala Leu Ile Val Asn Pro Tyr Asp Arg Asp Glu Val	
	400 405 410	
10	GCA GCT GCG CTG GAT CGT GCA TTG ACT ATG TCG CTG GCG GAA CGT ATT	1299
	Ala Ala Ala Leu Asp Arg Ala Leu Thr Met Ser Leu Ala Glu Arg Ile	
	415 420 425	
15	TCC CGT CAT GCA GAA ATG CTG GAC GTT ATC GTG AAA AAC GAT ATT AAC	1347
	Ser Arg His Ala Glu Met Leu Asp Val Ile Val Lys Asn Asp Ile Asn	
	430 435 440	
20	CAC TGG CAG GAG TGC TTC ATT AGC GAC CTA AAG CAG ATA GTT CCG CGA	1395
	His Trp Gln Glu Cys Phe Ile Ser Asp Leu Lys Gln Ile Val Pro Arg	
	445 450 455	
25	AGC GCG GAA AGC CAG CAG CGC GAT AAA GTT GCT ACC TTT CCA AAG CTT	1443
	Ser Ala Glu Ser Gln Gln Arg Asp Lys Val Ala Thr Phe Pro Lys Leu	
	460 465 470 475	
30	GCG	1446
	Ala	

(2) INFORMATION FOR SEQ ID NO: 2:

30

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 476 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

35

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

40	Met Thr Met Ser Arg Leu Val Val Val Ser Asn Arg Ile Ala Pro Pro	
	1 5 10 15	
	Asp Glu His Ala Ala Ser Ala Gly Gly Leu Ala Val Gly Ile Leu Gly	
	20 25 30	
45	Ala Leu Lys Ala Ala Gly Gly Leu Trp Phe Gly Trp Ser Gly Glu Thr	
	35 40 45	
50	Gly Asn Glu Asp Gln Pro Leu Lys Lys Val Lys Lys Gly Asn Ile Thr	
	50 55 60	
	Trp Ala Ser Phe Asn Leu Ser Glu Gln Asp Leu Asp Glu Tyr Tyr Asn	
	65 70 75 80	

Gln Phe Ser Asn Ala Val Leu Trp Pro Ala Phe His Tyr Arg Leu Asp
 85 90 95
 5 Leu Val Gln Phe Gln Arg Pro Ala Trp Asp Gly Tyr Leu Arg Val Asn
 100 105 110
 Ala Leu Leu Ala Asp Lys Leu Leu Pro Leu Leu Gln Asp Asp Asp Ile
 115 120 125
 10 Ile Trp Ile His Asp Tyr His Leu Leu Pro Phe Ala His Glu Leu Arg
 130 135 140
 Lys Arg Gly Val Asn Asn Arg Ile Gly Phe Phe Leu His Ile Pro Phe
 145 150 155 160
 15 Pro Thr Pro Glu Ile Phe Asn Ala Leu Pro Thr Tyr Asp Thr Leu Leu
 165 170 175
 Glu Gln Leu Cys Asp Tyr Asp Leu Leu Gly Phe Gln Thr Glu Asn Asp
 20 180 185 190
 Arg Leu Ala Phe Leu Asp Cys Leu Ser Asn Leu Thr Arg Val Thr Thr
 195 200 205
 25 Arg Ser Ala Lys Ser His Thr Ala Trp Gly Lys Ala Phe Arg Thr Glu
 210 215 220
 Val Tyr Pro Ile Gly Ile Glu Pro Lys Glu Ile Ala Lys Gln Ala Ala
 225 230 235 240
 30 Gly Pro Leu Pro Pro Lys Leu Ala Gln Leu Lys Ala Glu Leu Lys Asn
 245 250 255
 Val Gln Asn Ile Phe Ser Val Glu Arg Leu Asp Tyr Ser Lys Gly Leu
 35 260 265 270
 Pro Glu Arg Phe Leu Ala Tyr Glu Ala Leu Leu Glu Lys Tyr Pro Gln
 275 280 285
 40 His His Gly Lys Ile Arg Tyr Thr Gln Ile Ala Pro Thr Ser Arg Gly
 290 295 300
 Asp Val Gln Ala Tyr Gln Asp Ile Arg His Gln Leu Glu Asn Glu Ala
 305 310 315 320
 45 Gly Arg Ile Asn Gly Lys Tyr Gly Gln Leu Gly Trp Thr Pro Leu Tyr
 325 330 335
 Tyr Leu Asn Gln His Phe Asp Arg Lys Leu Leu Met Lys Ile Phe Arg
 50 340 345 350
 Tyr Ser Asp Val Gly Leu Val Thr Pro Leu Arg Asp Gly Met Asn Leu
 355 360 365

Val Ala Lys Glu Tyr Val Ala Ala Gln Asp Pro Ala Asn Pro Gly Val
 370 375 380

Leu Val Leu Ser Gln Phe Ala Gly Ala Ala Asn Glu Leu Thr Ser Ala
 5 385 390 395 400

Leu Ile Val Asn Pro Tyr Asp Arg Asp Glu Val Ala Ala Ala Leu Asp
 405 410 415

10 Arg Ala Leu Thr Met Ser Leu Ala Glu Arg Ile Ser Arg His Ala Glu
 420 425 430

Met Leu Asp Val Ile Val Lys Asn Asp Ile Asn His Trp Gln Glu Cys
 435 440 445

15 Phe Ile Ser Asp Leu Lys Gln Ile Val Pro Arg Ser Ala Glu Ser Gln
 450 455 460

Gln Arg Asp Lys Val Ala Thr Phe Pro Lys Leu Ala
 20 465 470 475

(2) INFORMATION FOR SEQ ID NO: 3:

- (i) SEQUENCE CHARACTERISTICS:
 25 (A) LENGTH: 25 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (iii) HYPOTHETICAL: YES
- (iii) ANTI-SENSE: NO
- 35 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

AAGCTTATGT TGCCATATAG AGTAG

25

40 (2) INFORMATION FOR SEQ ID NO: 4:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 24 base pairs
 (B) TYPE: nucleic acid
 45 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- 50 (iii) HYPOTHETICAL: YES
- (iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

GTAGTTGCCA TGGTGCAAAT GTTC

24

5 (2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 20 base pairs
 (B) TYPE: nucleic acid
 10 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

15 (iii) HYPOTHETICAL: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

20 AGCTCTGCAG TGAGGTACCA

20

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
 25 (A) LENGTH: 20 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

30 (ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: YES

35 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

GACGTCCTC CATGGTTCGA

20

(2) INFORMATION FOR SEQ ID NO: 7:

40 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 24 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 45 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: YES

50

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

GTACCCTGCA GTGTGACCCT AGAC

24

(2) INFORMATION FOR SEQ ID NO: 8:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 21 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (iii) HYPOTHETICAL: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

TCGATTCATA GAAGCTTAGA T 21

(2) INFORMATION FOR SEQ ID NO: 9:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 2207 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: double
 (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA to mRNA
- (iii) HYPOTHETICAL: NO
- (iii) ANTI-SENSE: NO
- (vi) ORIGINAL SOURCE:
 (A) ORGANISM: Solanum tuberosum
 (B) STRAIN: Kardal
- (ix) FEATURE:
 (A) NAME/KEY: CDS
 (B) LOCATION: 161..1906
- (ix) FEATURE:
 (A) NAME/KEY: misc_feature
 (B) LOCATION: 842..850
 (D) OTHER INFORMATION: /function= "putative glycosylationsite"
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

CTTTTCTGAG TAATAACATA GGCATTGATT TTTTTCATTA TAATAACACC TGCAAACATT 60

CCCATTGCCG GCATTCTCTG TTCTTACAAA AAAAAACATT TTTTGTTCATA CATAAATTAG 120

TTATGGCATC AGTATTGAAC CCTTTAACTT GTTATACAAT ATG GGT AAA GCT ATA 175

Met Gly Lys Ala Ile

1 5

	ATT TTT ATG ATT TTT ACT ATG TCT ATG AAT ATG ATT AAA GCT GAA ACT	223
	Ile Phe Met Ile Phe Thr Met Ser Met Asn Met Ile Lys Ala Glu Thr	
	10 15 20	
5	TGC AAA TCC ATT GAT AAG GGT CCT GTA ATC CCA ACA ACC CCT TTA GTG	271
	Cys Lys Ser Ile Asp Lys Gly Pro Val Ile Pro Thr Thr Pro Leu Val	
	25 30 35	
10	ATT TTT CTT GAA AAA GTT CAA GAA GCT GCT CTT CAA ACT TAT GGC CAT	319
	Ile Phe Leu Glu Lys Val Gln Glu Ala Ala Leu Gln Thr Tyr Gly His	
	40 45 50	
15	AAA GGG TTT GAT GCT AAA CTG TTT GTT GAT ATG TCA CTG AGA GAG AGT	367
	Lys Gly Phe Asp Ala Lys Leu Phe Val Asp Met Ser Leu Arg Glu Ser	
	55 60 65	
20	CTT TCA GAA ACA GTT GAA GCT TTT AAT AAG CTT CCA AGA GTT GTG AAT	415
	Leu Ser Glu Thr Val Glu Ala Phe Asn Lys Leu Pro Arg Val Val Asn	
	70 75 80 85	
25	GGT TCA ATA TCA AAA AGT GAT TTG GAT GGT TTT ATA GGT AGT TAC TTG	463
	Gly Ser Ile Ser Lys Ser Asp Leu Asp Gly Phe Ile Gly Ser Tyr Leu	
	90 95 100	
30	AGT AGT CCT GAT AAG GAT TTG GTT TAT GTT GAG CCT ATG GAT TTT GTG	511
	Ser Ser Pro Asp Lys Asp Leu Val Tyr Val Glu Pro Met Asp Phe Val	
	105 110 115	
35	GCT GAG CCT GAA GGC TTT TTG CCA AAG GTG AAG AAT TCT GAG GTG AGG	559
	Ala Glu Pro Glu Gly Phe Leu Pro Lys Val Lys Asn Ser Glu Val Arg	
	120 125 130	
40	GCA TGG GCA TTG GAG GTG CAT TCA CTT TGG AAG AAT TTA AGT AGG AAA	607
	Ala Trp Ala Leu Glu Val His Ser Leu Trp Lys Asn Leu Ser Arg Lys	
	135 140 145	
45	GTG GCT GAT CAT GTA TTG GAA AAA CCA GAG TTG TAT ACT TTG CTT CCA	655
	Val Ala Asp His Val Leu Glu Lys Pro Glu Leu Tyr Thr Leu Leu Pro	
	150 155 160 165	
50	TTG AAA AAT CCA GTT ATT ATA CCG GGA TCG CGT TTT AAG GAG GTT TAT	703
	Leu Lys Asn Pro Val Ile Ile Pro Gly Ser Arg Phe Lys Glu Val Tyr	
	170 175 180	
55	TAT TGG GAT TCT TAT TGG GTA ATA AGG GGT TTG TTA GCA AGC AAA ATG	751
	Tyr Trp Asp Ser Tyr Trp Val Ile Arg Gly Leu Leu Ala Ser Lys Met	
	185 190 195	
60	TAT GAA ACT GCA AAA GGG ATT GTG ACT AAT CTG GTT TCT CTG ATA GAT	799
	Tyr Glu Thr Ala Lys Gly Ile Val Thr Asn Leu Val Ser Leu Ile Asp	
	200 205 210	

	CAA	TTT	GGT	TAT	GTT	CTT	AAC	GGT	GCA	AGA	GCA	TAC	TAC	AGT	AAC	AGA	847
	Gln	Phe	Gly	Tyr	Val	Leu	Asn	Gly	Ala	Arg	Ala	Tyr	Tyr	Ser	Asn	Arg	
	215						220					225					
5	AGT	CAG	CCT	CCT	GTC	CTG	GCC	ACG	ATG	ATT	GTT	GAC	ATA	TTC	AAT	CAG	895
	Ser	Gln	Pro	Pro	Val	Leu	Ala	Thr	Met	Ile	Val	Asp	Ile	Phe	Asn	Gln	
	230					235					240					245	
	ACA	GGT	GAT	TTA	AAT	TTG	GTT	AGA	AGA	TCC	CTT	CCT	GCT	TTG	CTC	AAG	943
10	Thr	Gly	Asp	Leu	Asn	Leu	Val	Arg	Arg	Ser	Leu	Pro	Ala	Leu	Leu	Lys	
					250					255						260	
	GAG	AAT	CAT	TTT	TGG	AAT	TCA	GGA	ATA	CAT	AAG	GTG	ACT	ATT	CAA	GAT	991
15	Glu	Asn	His	Phe	Trp	Asn	Ser	Gly	Ile	His	Lys	Val	Thr	Ile	Gln	Asp	
				265					270						275		
	GCT	CAG	GGA	TCA	AAC	CAC	AGC	TTG	AGT	CGG	TAC	TAT	GCT	ATG	TGG	AAT	1039
	Ala	Gln	Gly	Ser	Asn	His	Ser	Leu	Ser	Arg	Tyr	Tyr	Ala	Met	Trp	Asn	
				280					285					290			
20	AAG	CCC	CGT	CCA	GAA	TCG	TCA	ACT	ATA	GAC	AGT	GAA	ACA	GCT	TCC	GTA	1087
	Lys	Pro	Arg	Pro	Glu	Ser	Ser	Thr	Ile	Asp	Ser	Glu	Thr	Ala	Ser	Val	
		295					300					305					
25	CTC	CCA	AAT	ATA	TGT	GAA	AAA	AGA	GAA	TTA	TAC	CGT	GAA	CTG	GCA	TCA	1135
	Leu	Pro	Asn	Ile	Cys	Glu	Lys	Arg	Glu	Leu	Tyr	Arg	Glu	Leu	Ala	Ser	
	310					315					320					325	
	GCT	GCT	GAA	AGT	GGA	TGG	GAT	TTC	AGT	TCA	AGA	TGG	ATG	AGC	AAC	GGA	1183
30	Ala	Ala	Glu	Ser	Gly	Trp	Asp	Phe	Ser	Ser	Arg	Trp	Met	Ser	Asn	Gly	
					330					335						340	
	TCT	GAT	CTG	ACA	ACA	ACT	AGT	ACA	ACA	TCA	ATT	CTA	CCA	GTT	GAT	TTG	1231
35	Ser	Asp	Leu	Thr	Thr	Thr	Ser	Thr	Thr	Ser	Ile	Leu	Pro	Val	Asp	Leu	
				345					350						355		
	AAT	GCA	TTC	CTT	CTG	AAG	ATG	GAA	CTT	GAC	ATT	GCC	TTT	CTA	GCA	AAT	1279
	Asn	Ala	Phe	Leu	Leu	Lys	Met	Glu	Leu	Asp	Ile	Ala	Phe	Leu	Ala	Asn	
			360					365					370				
40	CTT	GTT	GGA	GAA	AGT	AGC	ACG	GCT	TCA	CAT	TTT	ACA	GAA	GCT	GCT	CAA	1327
	Leu	Val	Gly	Glu	Ser	Ser	Thr	Ala	Ser	His	Phe	Thr	Glu	Ala	Ala	Gln	
		375					380					385					
45	AAT	AGA	CAG	AAG	GCT	ATA	AAC	TGT	ATC	TTT	TGG	AAC	GCA	GAG	ATG	GGG	1375
	Asn	Arg	Gln	Lys	Ala	Ile	Asn	Cys	Ile	Phe	Trp	Asn	Ala	Glu	Met	Gly	
	390					395					400					405	
	CAA	TGG	CTT	GAT	TAC	TGG	CTT	ACC	AAC	AGC	GAC	ACA	TCT	GAG	GAT	ATT	1423
50	Gln	Trp	Leu	Asp	Tyr	Trp	Leu	Thr	Asn	Ser	Asp	Thr	Ser	Glu	Asp	Ile	
					410					415					420		

	TAT AAA TGG GAA GAT TTG CAC CAG AAC AAG AAG TCA TTT GCC TCT AAT	1471
	Tyr Lys Trp Glu Asp Leu His Gln Asn Lys Lys Ser Phe Ala Ser Asn	
	425 430 435	
5	TTT GTT CCG CTG TGG ACT GAA ATT TCT TGT TCA GAT AAT AAT ATC ACA	1519
	Phe Val Pro Leu Trp Thr Glu Ile Ser Cys Ser Asp Asn Asn Ile Thr	
	440 445 450	
	ACT CAG AAA GTA GTT CAA AGT CTC ATG AGC TCG GGC TTG CTT CAG CCT	1567
10	Thr Gln Lys Val Val Gln Ser Leu Met Ser Ser Gly Leu Leu Gln Pro	
	455 460 465	
	GCA GGG ATT GCA ATG ACC TTG TCT AAT ACT GGA CAG CAA TGG GAT TTT	1615
15	Ala Gly Ile Ala Met Thr Leu Ser Asn Thr Gly Gln Gln Trp Asp Phe	
	470 475 480 485	
	CCG AAT GGT TGG CCC CCC CTT CAA CAC ATA ATC ATT GAA GGT CTC TTA	1663
	Pro Asn Gly Trp Pro Pro Leu Gln His Ile Ile Ile Glu Gly Leu Leu	
	490 495 500	
20	AGG TCT GGA CTA GAA GAG GCA AGA ACC TTA GCA AAA GAC ATT GCT ATT	1711
	Arg Ser Gly Leu Glu Glu Ala Arg Thr Leu Ala Lys Asp Ile Ala Ile	
	505 510 515	
25	CGC TGG TTA AGA ACT AAC TAT GTG ACT TAC AAG AAA ACC GGT GCT ATG	1759
	Arg Trp Leu Arg Thr Asn Tyr Val Thr Tyr Lys Lys Thr Gly Ala Met	
	520 525 530	
	TAT GAA AAA TAT GAT GTC ACA AAA TGT GGA GCA TAT GGA GGT GGT GGT	1807
30	Tyr Glu Lys Tyr Asp Val Thr Lys Cys Gly Ala Tyr Gly Gly Gly Gly	
	535 540 545	
	GAA TAT ATG TCC CAA ACG GGT TTC GGA TGG TCA AAT GGC GTT GTA CTG	1855
35	Glu Tyr Met Ser Gln Thr Gly Phe Gly Trp Ser Asn Gly Val Val Leu	
	550 555 560 565	
	GCA CTT CTA GAG GAA TTT GGA TGG CCT GAA GAT TTG AAG ATT GAT TGC	1903
	Ala Leu Leu Glu Glu Phe Gly Trp Pro Glu Asp Leu Lys Ile Asp Cys	
	570 575 580	
40	TAATGAGCAA GTAGAAAAGC CAAATGAAAC ATCATTGAGT TTTATTTTCT TCTTTTGTTA	1963
	AAATAAGCTG CAATGGTTTG CTGATAGTTT ATGTTTTGTA TTACTATTTC ATAAGGTTTT	2023
45	TGTACCATAT CAAGTGATAT TACCATGAAC TATGTCGTTT GGACTCTTCA AATCGGATTT	2083
	TGCAAAAATA ATGCAGTTTT GGAGAATCCG ATAACATAGA CCATGTATGG ATCTAAATTG	2143
	TAAACAGCTT ACTATATTAA GTAAAAGAAA GATGATTCCT CTGCTTTAAA AAAAAAAAAA	2203
50	AAAA	2207

5

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

	Met 1	Gly	Lys	Ala	Ile 5	Ile	Phe	Met	Ile	Phe 10	Thr	Met	Ser	Met	Asn 15	Met
15	Ile	Lys	Ala	Glu 20	Thr	Cys	Lys	Ser	Ile 25	Asp	Lys	Gly	Pro	Val 30	Ile	Pro
	Thr	Thr	Pro 35	Leu	Val	Ile	Phe	Leu 40	Glu	Lys	Val	Gln	Glu 45	Ala	Ala	Leu
20	Gln	Thr 50	Tyr	Gly	His	Lys	Gly 55	Phe	Asp	Ala	Lys	Leu 60	Phe	Val	Asp	Met
	Ser 65	Leu	Arg	Glu	Ser	Leu 70	Ser	Glu	Thr	Val	Glu 75	Ala	Phe	Asn	Lys	Leu 80
25	Pro	Arg	Val	Val	Asn 85	Gly	Ser	Ile	Ser	Lys 90	Ser	Asp	Leu	Asp	Gly 95	Phe
30	Ile	Gly	Ser	Tyr 100	Leu	Ser	Ser	Pro	Asp 105	Lys	Asp	Leu	Val	Tyr 110	Val	Glu
	Pro	Met	Asp 115	Phe	Val	Ala	Glu	Pro 120	Glu	Gly	Phe	Leu	Pro 125	Lys	Val	Lys
35	Asn	Ser 130	Glu	Val	Arg	Ala	Trp 135	Ala	Leu	Glu	Val	His 140	Ser	Leu	Trp	Lys
	Asn 145	Leu	Ser	Arg	Lys	Val 150	Ala	Asp	His	Val	Leu 155	Glu	Lys	Pro	Glu	Leu 160
40	Tyr	Thr	Leu	Leu	Pro 165	Leu	Lys	Asn	Pro	Val 170	Ile	Ile	Pro	Gly	Ser 175	Arg
45	Phe	Lys	Glu	Val 180	Tyr	Tyr	Trp	Asp	Ser 185	Tyr	Trp	Val	Ile	Arg 190	Gly	Leu
	Leu	Ala	Ser 195	Lys	Met	Tyr	Glu	Thr 200	Ala	Lys	Gly	Ile	Val 205	Thr	Asn	Leu
50	Val	Ser 210	Leu	Ile	Asp	Gln	Phe 215	Gly	Tyr	Val	Leu	Asn 220	Gly	Ala	Arg	Ala

	Tyr	Tyr	Ser	Asn	Arg	Ser	Gln	Pro	Pro	Val	Leu	Ala	Thr	Met	Ile	Val	
	225					230					235					240	
5	Asp	Ile	Phe	Asn	Gln	Thr	Gly	Asp	Leu	Asn	Leu	Val	Arg	Arg	Ser	Leu	
				245						250					255		
	Pro	Ala	Leu	Leu	Lys	Glu	Asn	His	Phe	Trp	Asn	Ser	Gly	Ile	His	Lys	
				260					265					270			
10	Val	Thr	Ile	Gln	Asp	Ala	Gln	Gly	Ser	Asn	His	Ser	Leu	Ser	Arg	Tyr	
			275					280					285				
	Tyr	Ala	Met	Trp	Asn	Lys	Pro	Arg	Pro	Glu	Ser	Ser	Thr	Ile	Asp	Ser	
		290					295					300					
15	Glu	Thr	Ala	Ser	Val	Leu	Pro	Asn	Ile	Cys	Glu	Lys	Arg	Glu	Leu	Tyr	
	305					310					315					320	
	Arg	Glu	Leu	Ala	Ser	Ala	Ala	Glu	Ser	Gly	Trp	Asp	Phe	Ser	Ser	Arg	
20				325						330					335		
	Trp	Met	Ser	Asn	Gly	Ser	Asp	Leu	Thr	Thr	Thr	Ser	Thr	Thr	Ser	Ile	
				340					345					350			
25	Leu	Pro	Val	Asp	Leu	Asn	Ala	Phe	Leu	Leu	Lys	Met	Glu	Leu	Asp	Ile	
			355					360					365				
	Ala	Phe	Leu	Ala	Asn	Leu	Val	Gly	Glu	Ser	Ser	Thr	Ala	Ser	His	Phe	
		370					375					380					
30	Thr	Glu	Ala	Ala	Gln	Asn	Arg	Gln	Lys	Ala	Ile	Asn	Cys	Ile	Phe	Trp	
	385					390					395					400	
	Asn	Ala	Glu	Met	Gly	Gln	Trp	Leu	Asp	Tyr	Trp	Leu	Thr	Asn	Ser	Asp	
35				405					410						415		
	Thr	Ser	Glu	Asp	Ile	Tyr	Lys	Trp	Glu	Asp	Leu	His	Gln	Asn	Lys	Lys	
				420					425					430			
40	Ser	Phe	Ala	Ser	Asn	Phe	Val	Pro	Leu	Trp	Thr	Glu	Ile	Ser	Cys	Ser	
		435						440					445				
	Asp	Asn	Asn	Ile	Thr	Thr	Gln	Lys	Val	Val	Gln	Ser	Leu	Met	Ser	Ser	
		450					455					460					
45	Gly	Leu	Leu	Gln	Pro	Ala	Gly	Ile	Ala	Met	Thr	Leu	Ser	Asn	Thr	Gly	
	465					470				475						480	
	Gln	Gln	Trp	Asp	Phe	Pro	Asn	Gly	Trp	Pro	Pro	Leu	Gln	His	Ile	Ile	
50				485					490						495		
	Ile	Glu	Gly	Leu	Leu	Arg	Ser	Gly	Leu	Glu	Glu	Ala	Arg	Thr	Leu	Ala	
				500					505						510		

Lys Asp Ile Ala Ile Arg Trp Leu Arg Thr Asn Tyr Val Thr Tyr Lys
 515 520 525
 Lys Thr Gly Ala Met Tyr Glu Lys Tyr Asp Val Thr Lys Cys Gly Ala
 5 530 535 540
 Tyr Gly Gly Gly Gly Glu Tyr Met Ser Gln Thr Gly Phe Gly Trp Ser
 545 550 555 560
 10 Asn Gly Val Val Leu Ala Leu Leu Glu Glu Phe Gly Trp Pro Glu Asp
 565 570 575
 Leu Lys Ile Asp Cys
 580
 15

(2) INFORMATION FOR SEQ ID NO: 11:

- 20 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 33 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
 25 (ii) MOLECULE TYPE: DNA (genomic)
 (iii) HYPOTHETICAL: YES
 30 (ix) FEATURE:
 (A) NAME/KEY: modified_base
 (B) LOCATION: 6
 (D) OTHER INFORMATION: /mod_base= i
 35 (ix) FEATURE:
 (A) NAME/KEY: modified_base
 (B) LOCATION: 15
 (D) OTHER INFORMATION: /mod_base= i
 40 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

GGYGGNMGMT TYRWNGARKT MTAYKRYTGG GAC

33

(2) INFORMATION FOR SEQ ID NO: 12:

- 45 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 26 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 50 (D) TOPOLOGY: linear
 (ii) MOLECULE TYPE: DNA (genomic)
 (iii) HYPOTHETICAL: YES

(ix) FEATURE:
 (A) NAME/KEY: modified_base
 (B) LOCATION: 3
 (D) OTHER INFORMATION: /mod_base= i

5

(ix) FEATURE:
 (A) NAME/KEY: modified_base
 (B) LOCATION: 6
 (D) OTHER INFORMATION: /mod_base= i

10

(ix) FEATURE:
 (A) NAME/KEY: modified_base
 (B) LOCATION: 9
 (D) OTHER INFORMATION: /mod_base= i

15

(ix) FEATURE:
 (A) NAME/KEY: modified_base
 (B) LOCATION: 12
 (D) OTHER INFORMATION: /mod_base= i

20

(ix) FEATURE:
 (A) NAME/KEY: modified_base
 (B) LOCATION: 15
 (D) OTHER INFORMATION: /mod_base= i

25

(ix) FEATURE:
 (A) NAME/KEY: modified_base
 (B) LOCATION: 21
 (D) OTHER INFORMATION: /mod_base= i

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

GTNCCNGGNG GNCGNTTYRW NGARKT

26

35

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 26 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

40

(ii) MOLECULE TYPE: DNA (genomic)

45

(iii) HYPOTHETICAL: YES

(ix) FEATURE:
 (A) NAME/KEY: modified_base
 (B) LOCATION: 3
 (D) OTHER INFORMATION: /mod_base= i

50

(ix) FEATURE:
 (A) NAME/KEY: modified_base
 (B) LOCATION: 9
 (D) OTHER INFORMATION: /mod_base= i

5

(ix) FEATURE:
 (A) NAME/KEY: modified_base
 (B) LOCATION: 12
 (D) OTHER INFORMATION: /mod_base= i

10

(ix) FEATURE:
 (A) NAME/KEY: modified_base
 (B) LOCATION: 15
 (D) OTHER INFORMATION: /mod_base= i

15

(ix) FEATURE:
 (A) NAME/KEY: modified_base
 (B) LOCATION: 18
 (D) OTHER INFORMATION: /mod_base= i

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

GGNGGYTGNS WNCGNYRNAG RTARTA

26

25

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 24 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

30

(ii) MOLECULE TYPE: DNA (genomic)

35

(iii) HYPOTHETICAL: YES

(ix) FEATURE:
 (A) NAME/KEY: modified_base
 (B) LOCATION: 1
 (D) OTHER INFORMATION: /mod_base= i

40

(ix) FEATURE:
 (A) NAME/KEY: modified_base
 (B) LOCATION: 7
 (D) OTHER INFORMATION: /mod_base= i

45

(ix) FEATURE:
 (A) NAME/KEY: modified_base
 (B) LOCATION: 19
 (D) OTHER INFORMATION: /mod_base= i

50

(ix) FEATURE:

(A) NAME/KEY: modified_base

(B) LOCATION: 22

(D) OTHER INFORMATION: /mod_base= i

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CLAIMS

1. A process for producing trehalose in plant cells capable of producing trehalase by growing plant cells having the genetic information required for the production of trehalose and trehalase, or cultivating a plant or a part thereof comprising such plant cells, characterised in that said plant cells are grown, or said plant or a part thereof, is cultivated in the presence of a trehalase inhibitor.
2. A process according to claim 1, wherein said plant cells have been genetically altered so as to contain a chimeric trehalose phosphate synthase gene in a plant expressible form, preferably wherein the trehalose phosphate synthase gene comprises an open reading frame encoding trehalose phosphate synthase from *E. coli* in plant expressible form, more preferably wherein the open reading frame encoding trehalose phosphate synthase from *E. coli* is downstream of the CaMV 35S RNA promoter or the potato patatin promoter.
3. A process according to claim 1 or 2, wherein a *Solanum tuberosum* plant is cultivated, preferably wherein said plant has micro-tubers.
4. A process according to claim 3, wherein said plant is cultivated *in vitro*.
5. A process according to any one of claims 1 to 4, wherein said trehalase inhibitor comprises validamycin A in a form suitable for uptake by said plant cells, said plant, or a part thereof, preferably wherein the concentration of validamycin A is between 100 nM and 10 mM, more preferably between 0.1 and 1 mM, in aqueous solution.
6. A process according to any one of claims 1 to 4, wherein said trehalase inhibitor comprises the 86kD protein of the cockroach (*Periplaneta americana*) in a form suitable for uptake by said plant cells, said plant, or a part thereof.

7. A process according to any one of claims 1 to 4, wherein said plant cells have been genetically altered to contain the genetic information for a trehalase inhibitor, preferably wherein the trehalase inhibitor is the antisense gene to the gene encoding the information for trehalase or wherein the trehalase inhibitor is the 86kD protein of the American cockroach (*Periplaneta americana*).

8. A process according to any one of claims 1 to 7, wherein a plant, or a part thereof, accumulates trehalose in an amount above 0.01 % (fresh weight).

9. A plant, or a part thereof, or plant cells, obtainable by a process according to any one of the claims 1 to 8, which contain trehalose in an amount above 0.01% (fresh weight), preferably wherein said plant, or a part thereof is a *Solanaceae* species, more preferably *Solanum tuberosum* or *Nicotiana tabacum*.

10. A plant part according to claim 9, which is a tuber or a micro-tuber.

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11. Tuber or micro-tubers of *Solanum tuberosum* containing trehalose.

12. Use of a plant, or plant part, according to claim 9 for extracting trehalose.

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13. Use of a plant, or plant part, according to claim 9 in a process of forced extraction of water from said plant or plant part.

14. A plant according to claim 9, which has an increased stress tolerance, preferably increased drought tolerance.

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15. A chimaeric plant expressible gene comprising in sequence a transcription initiation region obtainable from a gene, preferentially expressed in a plant part, particularly the patatin gene from *Solanum tuberosum*, a 5'-untranslated leader, an open reading frame encoding a trehalose phosphate synthase activity, and downstream of said open

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reading frame a transcriptional terminator region, preferably wherein said transcriptional terminator region is obtainable from the proteinase inhibitor-II gene of *Solanum tuberosum*.

5 16. A vector comprising a chimaeric plant expressible gene according to claim 15.

17. A recombinant plant genome comprising a chimaeric gene according to claim 16.

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18. A plant cell having a recombinant genome according to claim 17.

19. A plant or a part thereof, consisting essentially of cells according to claim 18, wherein said plant is *Solanum tuberosum*.

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20. A plant part according to claim 19, which is a tuber or a micro-tuber.

21. A process for obtaining trehalose, comprising the steps of growing
20 plant cells according to claim 18, or cultivating a plant according to claim 19, or cultivating a plant part according to any one of claims 19 or 20, extracting trehalose from said plant cells, plants or parts.

22. A process for obtaining trehalose, comprising the steps of
25 producing trehalose in plant cells, a plant or a part thereof, according to a process of any one of claims 1 to 8, and separating or extracting trehalose from said plant cells, plant or part thereof.

ABSTRACT

The invention provides a process for producing trehalose in plant cells capable of producing trehalase by growing plant cells having the genetic information required for the production of trehalose and
5 trehalase, or cultivating a plant or a part thereof comprising such plant cells, characterised in that said plant cells are grown, or said plant or a part thereof, is cultivated in the presence of a trehalase inhibitor.